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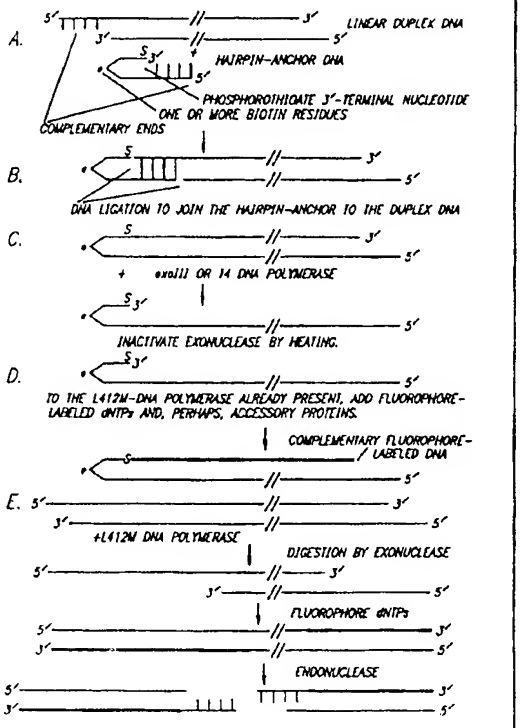
(54) Title: SYNTHESIS OF FLUOROPHORE-LABELED DNA

(57) Abstract

Sequencing methods and methods for synthesizing DNA probes using mutant bacteriophage T4 DNA polymerases which have increased ability to incorporate modified nucleotides for the synthesis of long or short chains of complementary, modified, e.g., fluorophore-labeled DNA. In general, the mutant T4 DNA polymerases retain 3'→5' exonuclease activity; hence, reduction or elimination of 3'→5' exonuclease activity is not a prerequisite for efficient synthesis of a complementary fluorophore-labeled or other modified DNA. In fact, retention of 3'→5' exonuclease activity increases accuracy of DNA replication, because these exonucleases proofread or edit the product of DNA replication.



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SYNTHESIS OF FLUOROPHORE-LABELED DNA

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to the fields of biology and chemistry. More particularly, the present invention is directed to methods for use in sequencing deoxyribonucleic acid (hereinafter referred to as "DNA") and for labeling DNA probes.

BACKGROUND OF THE INVENTION

Although a substantial amount of research has been directed to the development of sequencing methods, a limitation of current day techniques is that sequence information is obtained in units of only 400 to 600 nucleotides. For genome sequencing projects, such as sequencing the human genome, it would be inefficient to fit together such small units of sequence information. Longer units of sequence information would also be required in order to sequence through many repeated DNA sequences.

One proposed method to increase the length of sequence information is the single molecule sequencing method [U.S. Patent 4,962,037; Jett, J. H., *et al.*, *J. Biomolecular Structure & Dynamics*, 7:301-309 (1989); Ambrose, *et al.*, *Ber. Bunseniges Phys. Chem.*, 97:1535 (1993)]. For the single molecule sequencing method, a DNA polymerase is used to synthesize a complementary DNA with fluorophore-labeled deoxynucleoside triphosphates (fluorophore

dNTPs). Each of the four fluorophore dNTPs has a unique fluorophore tag that can be used to identify the nucleotide. A single fluorophore-labeled DNA is then immobilized in a flow cell and subjected to exonuclease digestion. A flow system carries each released fluorophore-labeled deoxynucleoside monophosphate (fluorophore dNMP) to a highly sensitive fluorescence detector capable of single molecule detection. The order of the fluorophore dNMPs detected gives the sequence. Because *in vitro* fluorophore-labeled DNA synthesized in this manner may be tens of thousands of nucleotides in length, this method will be useful in providing long sequence information.

The single molecule sequencing method has two primary enzymatic components. The first enzymatic component is employed in the synthesis of the complementary fluorophore-labeled DNA, synthesis being achieved by DNA polymerase-mediated incorporation of fluorophore-labeled nucleotides. The second enzymatic component is involved in digestion of the fluorophore-labeled DNA to release fluorophore dNMPs.

In principle, DNA polymerases from a variety of organisms would appear to have the potential to be used in *in vitro* reactions for the synthesis of complementary, fluorophore-labeled DNA. In practice, few DNA polymerases have been found to be suitable for this purpose. Synthesis of the complementary, fluorophore DNA requires first that the DNA polymerase have the ability to incorporate the fluorophore nucleotide. Second, the DNA polymerase must then be able to extend the fluorophore-labeled terminal nucleotide by addition of the next complementary fluorophore nucleotide. Incorporation of fluorophore nucleotides and extension of a fluorophore-labeled terminus are steps that are discriminated against by most DNA polymerases. A third requirement is that DNA replication must be accurate so that a faithful complementary fluorophore-labeled DNA is synthesized.

Methods for the synthesis of long fluorophore-labeled DNA can also be used to make shorter labeled DNAs, to be used as probes. DNA probes are used to identify chromosomes, locate genes and mRNA, *etc.* These methods can also

be used to synthesize biotin-labeled DNA, DIG-labeled DNA, *etc.*, which rely on the enzymatic incorporation into DNA of a labeled or modified nucleotide. "DIG" is the abbreviation of digoxigenin. For the biotin- and DIG-labeled DNAs, biotin- or DIG-labeled nucleotides are used; a fluorophore-dNTP is used for the synthesis of fluorophore-labeled DNA.

Another deficiency in current DNA sequencing methods is speed. The single molecule sequencing method has the potential to increase sequencing speed to 10 or more nucleotides per second [U.S. Patent 4,962,037; Jett, J. H., *et al.*, *J. Biomolecular Structure & Dynamics*, 7:301-309 (1989)]. Another method that has the potential to increase speed is mass spectrometry [Chen, C. H., *et al.*, *SPEI* **2386**:1322 (1995)]. Presently, a mass spectrometric method has been reported to sequence a 35-nucleotide oligomer in a few seconds. A limitation of mass spectrometry is that only short DNAs can be sequenced. Longer DNAs can be sequenced by mass spectrometry if the differences in mass between the four nucleotides can be increased. One way to increase differences in the mass of nucleotides is to use modified nucleotides, hence, synthesis of a complementary DNA with modified nucleotides may be the means to make mass spectrometry a useful sequencing method.

It is an object of the present invention to provide compositions and methods which do not suffer from all the drawbacks of the prior art.

SUMMARY OF THE INVENTION

In accordance with the present invention, there are provided improved DNA polymerases and methods for synthesizing DNA molecules with modified nucleotides using these improved DNA polymerases. These improved DNA polymerases have increased intrinsic processivity and increased ability to synthesize a complementary DNA (*e.g.*, from a DNA template) using a wide variety of modified nucleotides. For example, these improved DNA polymerases can be novel native DNA polymerases with increased processivity compared to known DNA polymerases. The improved DNA polymerases can also be mutant

DNA polymerases which possess increased intrinsic processivity compared to their native DNA polymerase counterparts. The resulting modified DNA products can be used in a variety of applications including, but not limited to, synthesis of DNA probes and DNA sequencing. In a preferred embodiment, the methods use mutant bacteriophage T4 DNA polymerases which have increased ability to synthesize accurately short or long chains of complementary, modified, *e.g.*, fluorophore-labeled DNA. In general, the mutant T4 DNA polymerases retain 3'→5' exonuclease activity; hence, reduction or elimination of 3'→5' exonuclease activity is not a prerequisite for efficient synthesis of a fluorophore-labeled complementary DNA. In fact, retention of 3'→5' exonuclease activity increases accuracy of DNA replication, because the exonuclease activity proofreads or edits the product of DNA replication.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention may be better understood with reference to the accompanying drawings, in which:

Figs. 1A-1F depict the structure of exemplary fluorophore nucleotides useful in the practice of the invention;

Figs. 2A and 2B depict the DNA sequencing gels which demonstrate the superior ability of the L412M-DNA polymerase to synthesize complementary fluorophore-labeled DNAs (2A) and plasmid DNA with Rhodamine-dUTP by wild type and L412M-DNA polymerases (2B);

Fig. 3A depicts the DNA sequencing gels which demonstrate that the L412M-DNA polymerase is superior to Sequenase in the synthesis of complementary fluorophore-labeled DNAs; Fig. 3B depicts the gels which demonstrate the importance of glycerol in the reaction mixture;

Fig. 4 depicts the steps of an exemplary procedure for synthesis of complementary fluorophore-labeled DNAs for single-molecule DNA sequencing; and

Fig. 5 illustrates the synthesis of biotin-, DIG- and fluorophore-labeled probes.

DETAILED DESCRIPTION OF THE INVENTION

Although naturally-occurring DNA polymerases are in general unsuitable for synthesis of long complementary chains of fluorophore-labeled DNA, mutant DNA polymerases identified by genetic selection have properties which allow for the efficient synthesis of a complementary, fluorophore-labeled DNA. Three types of DNA polymerase modifications are predicted to improve the ability of DNA polymerases to synthesize a complementary fluorophore-labeled DNA: (1) reduction or loss of 3'→5' exonuclease activity present in many naturally-occurring DNA polymerases; (2) increased ability to incorporate fluorophore-labeled nucleotides; and (3) increased ability to extend fluorophore-labeled DNA. Elimination or reduction of 3'→5' exonuclease activity would be expected to prevent removal of incorporated fluorophore-labeled nucleotides, while increased incorporation and extension of fluorophore-labeled nucleotides would allow for efficient synthesis of fluorophore-labeled complementary DNAs. Loss of 3'→5' exonuclease activity, however, would reduce the accuracy of DNA replication. Thus, an ideal DNA polymerase for synthesis of fluorophore DNA would be an enzyme which retains all or some 3'→5' exonucleolytic proofreading activity in order to achieve accurate synthesis, but has increased ability to incorporate fluorophore nucleotides consecutively compared to wild-type DNA polymerase.

The invention is the discovery of variant DNA polymerases that can incorporate modified nucleotides used to synthesize DNA for single molecule sequencing, for DNA probes, and for mass spectrometry sequencing. Reaction conditions with the variant DNA polymerases have also been developed. The essence of the preferred embodiment is that variant T4 DNA polymerases with increased intrinsic processivity have increased ability to synthesize a complementary DNA with a variety of modified nucleotides. The resulting modified DNAs can be used in a variety of applications, but not limited to, DNA

sequencing including single molecule and mass spectrometry methods, and DNA probes.

In accordance with one preferred embodiment of the present invention, there is employed a bacteriophage T4 mutant DNA polymerase with increased ability to synthesize complementary fluorophore-labeled DNAs. An exemplary T4 mutant DNA polymerase suitable for use in accordance with the present invention is the L412M-DNA polymerase. The L412M-DNA polymerase is different from the wild type T4 DNA polymerase by having a methionine residue in place of a leucine residue at position 412. The identification of the L412M-DNA polymerase by genetic selection has been described in U.S. patent application Serial No. 08/101,593 filed August 2, 1993, and Stocki, S.A., *et al.*, *J. Mol. Biol.*, **254**:15-28 (1995), these disclosures are hereby incorporated by reference in their entirety.

Biochemical studies demonstrate that the L412M-DNA polymerase retains 3'→5' exonuclease activity, is more efficient in primer-extension, and has greater intrinsic processivity. Processivity is defined as the number of enzymatic steps carried out per enzyme encounter with the DNA substrate. Intrinsic processivity is defined as the processivity of the DNA polymerase alone without the addition of accessory proteins. The L412M-DNA polymerase also has greater ability to bind modified primer termini as demonstrated for fluorophore-, biotin- and DIG-modified primer termini and for primer termini with the base analog, 2-aminopurine.

The increased intrinsic processivity of the L412M-DNA polymerase is the distinguishing characteristic of this variant DNA polymerase which allows the enzyme to more efficiently incorporate modified nucleotides and to extend primer-termini with primers containing modified nucleotides. Although DNA polymerase accessory proteins enhance DNA polymerase processivity, the DNA polymerase intrinsic processivity determines if the DNA polymerase will be able to form an active DNA:DNA polymerase complex. Thus, enhanced processivity

conferred by accessory proteins is secondary to the intrinsic processivity of the DNA polymerase.

Bacteriophage T4 DNA polymerase is a member of a large group of protein sequence related DNA polymerases called Family B DNA polymerases [Braithwaite, D. K., *et al.*, *Nucl. Acids Res.*, **21**:787-802 (1993)]. The L412M amino acid substitution resides in a highly conserved DNA polymerase motif called Motif A [Delarue, M., *et al.*, *Protein Eng.*, **3**:461-467 (1990)]. Thus, amino acid substitutions in the Motif A sequence in other family B DNA polymerases may convert these DNA polymerases into enzymes with enhanced ability to extend primer-termini, with greater intrinsic processivity, and with greater ability to synthesize complementary DNAs with fluorophore-labeled nucleotides or with other modified nucleotides.

Similarly, other modifications to motif A and to other regions identified by genetic selection produce mutant DNA polymerases with properties advantageous for increased incorporation of fluorophore nucleotides which include increased processivity and increased extension of modified primer termini.

The following amino acid substitutions produce mutant DNA polymerases with properties similar to those of the L412M-DNA polymerase. These polymerases were initially identified and isolated by genetic selection described in Stocki, S.A., *et al.*, *J. Mol. Biol.*, 254:15-28 (1995). The DNA polymerases with asterisks (*) are now under active study, and have so far been shown to be like the L412M-DNA polymerase, in having increased ability to synthesize fluorophore-labeled DNA.

(*) Q380K (lysine substituted for glutamine at position 380)

(*) E395K (lysine substituted for glutamate at position 395)

(*) E743K (lysine substituted for glutamate at position 743)

M725I (isoleucine substituted for methionine at position 725)

M725V (valine substituted for methionine at position 725)

S756P (proline substituted for serine at position 756)

L771F (phenylalanine substituted for leucine at position 771)
L771H (histidine substituted for leucine at position 771)
[L771+V] (valine inserted following leucine 771)
[L771+D] (aspartate inserted following leucine 771)
V355A (alanine inserted for valine at position 355)

Other suitable DNA polymerases, besides T4 polymerase and/or the above amino acid substitutions, and native, artificially mutagenized or mutant polymerases may be identified and isolated by the genetic selection method described in Stocki, S.A., *et al.*, *id.* The selected polymerases may then be further selected based on their increased intrinsic processivity, using the methods described below, such as based on their increased ability to incorporate fluorophore and other bulky nucleotides in synthesizing complementary DNA. The preferred DNA polymerases, *e.g.*, mutant DNA polymerases, are characterized by having increased ability to extend primers and increased intrinsic processivity relative to the native polymerases, while retaining 3'→5' exonuclease activity. The preferred DNA polymerases may be novel native DNA polymerases with increased intrinsic processivity compared to known DNA polymerases. The more preferred polymerases further have the ability to synthesize long DNAs with normal dNTPs without dissociation. Once the sequence of a polymerase is known, it can be synthetically produced, *e.g.*, through cloning and recombinant technology using methods known in the art, such as described in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 2d ed., 1989) and Ausubel, F. M., *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates, New York (1993).

While the present invention is not limited to any particular theory, it is proposed that the mechanism for improved incorporation of modified nucleotides is due to amino acid substitutions that increase stabilization of DNA in the polymerase active center. Thus, any amino acid substitutions that increase stabilization of DNA in the polymerase active center have the potential to

produce a mutant DNA polymerase with increased ability to incorporate modified nucleotides, *e.g.*, to synthesize fluorophore-labeled DNA. These amino acid substitutions are now identified by genetic selection. In the future, structural information from DNA polymerase-DNA complexes may provide this information. These studies are with bacteriophage T4 DNA polymerase, but other DNA polymerases with amino acid changes that increase stability of DNA in the polymerase active center would also likely have increased ability to incorporate fluorophore and other bulky nucleotides.

Combinations of amino acid changes are also of interest as multiply mutant DNA polymerases may demonstrate a further increase in the ability to incorporate modified, *e.g.*, fluorophore nucleotides. For example, the E395K+L412M-DNA polymerase, the L412M+E743K-DNA polymerase, the E395K+L412M+E743K-DNA polymerase, and the Q380K+L412M+E743K-DNA polymerase are of interest.

Some Family B DNA polymerases are used commercially, *e.g.* the Vent (commercially available from New England BioLabs, Inc., Beverly, Massachusetts) and Pfu DNA polymerases. Unlike the T4 DNA polymerases, these enzymes are thermally stable. These enzymes have the conserved leucine residue in the motif A sequence, and substitution of a methionine residue for this conserved leucine or other amino acid substitutions in the Motif A sequence may allow these enzymes to be used in synthesizing modified, *e.g.*, fluorophore-labeled DNA.

Pursuant to the present invention, it has been determined that contrary to expectations 3'→5' exonuclease activity may be an asset in single molecule sequencing methods. The 3'→5' exonuclease activity of DNA polymerases functions to remove misincorporated nucleotides. If 3'→5' exonuclease activity is reduced, incorrect nucleotides at the primer-terminus cannot be removed. Because these mismatched primer-termini are poor substrates for further extension, further elongation of the DNA chain is prevented. A mutant DNA polymerase with increased ability to incorporate fluorophore nucleotides

consecutively but retaining 3'→5' exonuclease activity has been found to be a particularly useful enzyme for synthesis of fluorophore-labeled complementary DNA.

For DNA probe synthesis, the L412M-DNA polymerase and an exonuclease deficient form of the L412M-DNA polymerase are useful. Less accuracy is required for the synthesis of the shorter fluorophore-labeled DNA probes. Exonuclease deficiency was found to improve incorporation of some fluorophore and other modified nucleotides. Specifically, the triply mutant D112A+E114A+L412M-DNA polymerase, where the D112A and E114A amino acid substitutions remove most but not all of the 3'→5' exonuclease activity, was found to have improved incorporation of fluorophore, biotin, DIG and other modified nucleotides.

The wild type bacteriophage T4 DNA polymerase gene has been cloned and the protein product expressed [Lin, T.-C., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **84**:7000-7004 (1987); U.S. Patent 4,935,361]. Standard oligonucleotide-directed mutagenesis procedures were used to construct the L412M-DNA polymerase mutant gene for expression of large quantities of the mutant L412M-DNA polymerase. Large amounts of the L412M-DNA polymerase have been purified by a previously described method [Reha-Krantz, L. J., *et al.*, *J. Virol.*, **67**:60-66]. Using these same procedures, a large amount of exonuclease-deficient form of the L412M mutant, the triply-mutant D112A+E114A+L412M DNA polymerase, has been produced. The Q380K-, E395K- and E743-DNA polymerases were also constructed similarly.

In accordance with one aspect of the invention, there are provided methods for synthesizing long chains of complementary fluorophore-labeled DNA. The methods employ variant (mutant) DNA polymerases, characterized by having increased ability to extend primers and increased intrinsic processivity relative to native T4 polymerase, while retaining 3'→5' exonuclease activity. For example, the mutant enzyme L412M-DNA polymerase differs from the wild type T4 DNA polymerase by having increased ability to extend primers and by having

increased intrinsic processivity; however, like the wild type T4 DNA polymerase, the L412M-DNA polymerase retains an active 3'→5' exonuclease activity. Because the L412M-DNA polymerase is a derivative of the highly accurate T4 DNA polymerase and because the 3'→5' exonuclease activity is retained, DNA products synthesized by the L412M-DNA polymerase are accurate. The increased ability to extend primers and enhanced intrinsic processivity are conferred by a methionine amino acid substitution at position 412 in the T4 DNA polymerase in place of the leucine residue. The L412M-DNA polymerase, by virtue of its new properties, also has improved ability to incorporate other modified nucleotides and thus to synthesize other types of modified DNA. For example, the L412M-DNA polymerase has been used to incorporate biotin-dCMP to make biotin-labeled DNA. Other amino acid substitutions, Q380K, E395K, E743K and others noted above confer similar properties.

In accordance with another aspect of the invention, there are provided methods which are directed at implementing the single molecule sequencing method. The synthetic component of this method requires the synthesis of a complementary fluorophore-labeled DNA by a DNA polymerase. Methods to direct synthesis to one strand of the duplex DNA are described. These methods, although useful to the single molecule sequencing methods, may also be of use to other applications which require the synthesis of long chains of fluorophore-labeled DNA.

Several mutant T4 DNA polymerases identified by genetic selection were tested for their ability to synthesize complementary fluorophore-labeled DNAs. In addition to the L412M-DNA polymerase (methionine for leucine at position 412), two mutant T4 DNA polymerases with substantially reduced 3'→5' exonuclease activity were also tested: D112A+E114A (alanine substitutions for aspartate at position 112 and glutamate at position 114), and D219A (alanine in place of aspartate at position 219). Biochemical characterizations of the 3'→5' exonuclease deficient enzymes have been previously reported [Reha-Krantz, L.

J., *et al.*, *J. Biol. Chem.*, **268**:27100-27108 (1993)]. The partially exonuclease deficient G255S-DNA polymerase was also tested [Stocki, S.A., *et al.*, *J. Mol. Biol.*, **254**:15-28 (1995)].

In addition, a modified bacteriophage T7 DNA polymerase, Sequenase Version 2.0, was tested. Sequenase has at least two biochemical properties which might be expected to enable this enzyme to efficiently incorporate fluorophore nucleotides. One potentially advantageous property is its high processivity due to the presence of the accessory protein, thioredoxin, as part of the T7 DNA polymerase complex. Another potentially advantageous property is the elimination of 3'→5' exonuclease activity.

Comparisons of the mutant T4 DNA polymerases with Sequenase demonstrated that one of the mutant T4 DNA polymerases, the L412M-DNA polymerase, was superior to Sequenase in synthesizing complementary fluorophore-labeled DNAs. Since the L412M-DNA polymerase retained 3'→5' exonuclease activity while Sequenase and the T4 D112A+E114A and D219-DNA polymerases did not, 3'→5' exonuclease deficiency is not required for synthesis of long chains of complementary fluorophore-labeled DNA. These comparisons also demonstrate that increased ability to extend primers and enhanced intrinsic processivity are useful properties for the synthesis of fluorophore DNAs, because these are properties which distinguish the mutant L412M-DNA polymerase from the wild-type enzyme. Although Sequenase is also processive by virtue of association with thioredoxin, the processivity of the L412M-DNA polymerase differs since the methionine substitution for leucine 412 increases the <u>intrinsic</u> processivity of the DNA polymerase which is independent of accessory processivity proteins. The L412M has high intrinsic processivity which is increased in the presence of association of the DNA polymerase with accessory proteins.

An additional requirement in the synthesis component of the single molecule sequencing method is that complementary fluorophore-labeled products be synthesized with high fidelity. Wild type T4 DNA polymerase is one of the

most accurate DNA polymerases with an error frequency of about 10^{-8} errors/base pair [Kunkel, T. A., *et al.*, *J. Biol. Chem.*, 259:1539-1545 (1984)]. The L412M-DNA polymerase is about five- to about ten-fold less accurate.

Studies of the accuracy of DNA replication by the L412M-DNA polymerase with fluorophore dNTPs suggest that the L412M-DNA polymerase accurately incorporates fluorophore-labeled nucleotides. The L412M-DNA polymerase retains 3'→5' exonuclease activity which acts to proofread misincorporated nucleotides. Sequenase lacks 3'→5' exonuclease activity and, thus, has lower DNA replication fidelity than the T4 L412M-DNA polymerase. Furthermore, the lack of 3'→5' exonuclease activity in Sequenase may be the reason why Sequenase is less efficient than the T4 L412M-DNA polymerase in synthesizing long chains of fluorophore-labeled DNA.

DNA polymerases in general cannot efficiently extend mismatched primer-termini. The 3'→5' exonuclease activity acts to repair mismatched primer-termini and thus converts a primer-terminus that is only poorly extendable by a DNA polymerase to a correctly base-paired primer-terminus which is more readily extendable. For Sequenase, which does not have an active 3'→5' exonuclease activity, misincorporated nucleotides may result in mismatched primer-termini which cannot be extended; this may result in premature termination of synthesis. The T4 L412M-DNA polymerase, because of its 3'→5' exonuclease activity, can correct mismatched primer-termini, thereby improving the fidelity of DNA replication. This activity also prevents premature termination of replication. Thus, the DNA polymerase 3'→5' exonuclease activity appears to be an asset by allowing more accurate DNA replication and synthesis of longer products.

In accordance with another aspect of the invention, the T4 L412M-DNA polymerase is employed in combination with another DNA polymerase. In one embodiment, Sequenase is employed in combination with L412M-DNA polymerase. Sequenase is processive and this enzyme was second in efficiency in synthesizing fluorophore-labeled DNAs to the T4 L412M-DNA polymerase.

A combination of Sequenase and the L412M-DNA polymerase may in some instances realize the best attributes of both enzymes. Another possible combination is L412M-DNA polymerase and an exonuclease-deficient form of the polymerase (for example, the multiple mutant D112A+E114A+L412M-DNA polymerase). Yet another combination employs the L412M-DNA polymerase and a thermostable DNA polymerase (such as Vent or Vent modified to resemble the properties of the L412M-DNA polymerase).

In accordance with another aspect of the invention, the L412M-DNA polymerase is employed in the synthesis of fluorophore-labeled or other labeled DNAs to be used as probes. DNA probes are typically a few hundred to a few thousand nucleotides in length, with one nucleotide partially or fully substituted by a fluorophore-labeled nucleotide. When the DNA probes are added to the assay system, specific interaction between the DNA probe and the target DNA or RNA is observed due to base pairing between the probe and target DNA or RNA. In order to optimize fluorescence intensity of fluorescent-labeled probes, it is often appropriate to adjust the extent of fluorophore substitution. Instead of 100% fluorophore nucleotide in place of a standard dNTP, a mixture of fluorophore-dNTP and unmodified dNTP is used, with the optimum mixture for any given probe being determined by experiment to see what extent of fluorophore-nucleotide substitution gives the highest fluorescence. In addition to fluorophore nucleotides, this approach for making labeled probes has been successfully employed using other labeled nucleotides, such as biotin-labeled dUTP and biotin-labeled dCTP, and DIG-labeled dCTP.

For purposes of preparing probes and for use with some modified nucleotides, an exonuclease deficient version of the L412M-DNA polymerase may have advantages. For example, the D112A+E114A+L412M-DNA polymerase, while not optimal for use in DNA sequencing, may have particular utility in preparing probes using fluorophore-labeled or other modified nucleotides. Probes are shorter and a population of probes is a modal distribution, not likely to include more than a few copies of the same "mistake"

in synthesis. Moreover, even a few mistakes would not prevent the probes from basepairing with the target DNA or RNA. However, for single molecule DNA sequencing single molecules are sequenced, so essentially 100% accuracy is required in those uses.

Temperature is an important parameter in the synthesis of fluorophore DNA. Although synthesis of fluorophore DNA is observed at room temperature, a higher temperature of 42°C increases replication past secondary structures in the template DNA. The inclusion of 16-18% glycerol in the reactions also assists in the incorporation of modified nucleotides.

The invention may be better understood with reference to the accompanying examples, which are intended for purposes of illustration only and should not be construed as in any sense limiting the scope of the invention as defined in the claims appended hereto.

EXAMPLES

Example 1

Synthesis of complementary fluorophore-labeled DNA was tested using the following procedure. The DNA template was a single-stranded circular DNA of approximately 7000 nucleotides. The template was primed with a single, ^{32}P-labeled complementary oligonucleotide. The test was to measure how far various DNA polymerases could extend the labeled primer when fluorophore-labeled dNTPs were supplied in place of the standard unmodified dNTPs. After the reaction mixtures were incubated, the primer-extension products were separated by electrophoresis on standard DNA sequencing gels. The size of the reaction products was revealed after exposure of the gels to X-ray film.

Six fluorophore-labeled nucleotides were tested. Fluorescein-12-2'-deoxy-uridine-5'-triphosphate (Fig. 1A) and fluorescein-15-2'-deoxy-adenine-5'-triphosphate (Fig. 1B) were purchased from Boehringer Mannheim (Indianapolis, Indiana). Rhodamine-12-dUTP (Fig. 1C), rhodamine-dATP (Fig. 1D),

rhodamine-dCTP (Fig. 1E), and fluorescein-dCTP (Fig. 1F) were provided by Life Technologies Incorporated, Bethesda, Maryland.

The reaction mixtures contained 67 mM Tris-HCl (pH 8.8), 16.7 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, 6.7 mM $MgCl_2$, and 167 μg/ml bovine serum albumin. The polymerase concentrations were 0.15 - 0.3 pmol/ml and the DNA concentration was 7.5 fmol/ml. Nucleotide concentrations, dNTP and fluorophore-labeled dNTPs, were each at 80 μM. The reaction volume was 10 μl. Reactions were incubated at 37°C for the indicated times.

Fluorescein-12-dUTP (Fig. 1A), rhodamine-12-dUTP (Fig. 1C), rhodamine-dATP (Fig. 1D), rhodamine-dCTP (Fig. 1E) and fluorescein-dCTP (Fig. 1F) were incorporated to variable extents by the DNA polymerases tested. The mutant T4 DNA polymerases and Sequenase performed better than the wild type T4 DNA polymerase. Fluorescein-15-dATP (Fig. 1B) was poorly incorporated by the DNA polymerases tested and was, thus, found less suitable for use in the synthesis of fluorophore-labeled DNA.

The single molecule sequencing method requires that two or more fluorophore-nucleotides be substituted for standard, unmodified nucleotides. Reactions with pairwise combinations of the fluorophore nucleotides provide useful information about the efficacy of various DNA polymerases for the synthesis of fluorophore-labeled DNAs. Reactions with the exonuclease deficient T4 D219A-DNA polymerase, the T4 L412M- and G255S-DNA polymerases, and the Klenow fragment of *E. coli* DNA pol I are shown in Fig. 2A. Reactions were incubated for 18.5 hours at 37°C. Reactions in lanes a-d contain rhodamine-dATP (Fig. 1D) in place of dATP. Reactions in lanes e-h contain rhodamine-dCTP (Fig. 1E) in place of dCTP. Reactions in lanes i-l contain the combination of rhodamine-dATP and rhodamine-dCTP in place of dATP and dCTP. Under all conditions, the longest complementary fluorophore-labeled products were synthesized by the T4 L412M-DNA polymerase which has increased intrinsic processivity (Fig. 2A, lanes b, f and j). The G255S-DNA polymerase (Fig. 2, lanes c, g and k) was not as efficient. The exonuclease

deficient DNA polymerases, the D219A-DNA polymerase (Fig. 2, lanes a, e and i) and the D112A+E114A-DNA polymerase (data not shown) were also not as efficient as the L412M-DNA polymerase. The Klenow fragment reactions were also less efficient (lanes d, h and l).

Reaction products were shorter when two fluorophore nucleotides were used (Fig. 2A, lanes i-l). It is likely that the size of the products with rhodamine-dATP and dCTP is an underestimate of the ability of the enzymes to synthesize fluorophore-labeled DNA, because the fluorophore is attached to the bases at hydrogen bonding positions; attachment at hydrogen bonding positions affects base pairing. Nucleotides with modifications that do not affect base pairing positions are expected to be more efficiently incorporated.

The next test was to determine if the L412M-DNA polymerase could synthesize a full-length copy of plasmid DNA if rhodamine-12-dUTP (Fig. 1C) was substituted for TTP. The reaction conditions were further optimized and contained 18% glycerol, 67 mM Tris-HCl (pH 8.8), 16.7 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, 6.7 mM $MgCl_2$, and 167 μg/ml BSA. Rhodamine-dUTP and dATP, dCTP, and dGTP were at 200 μM. There was a ten-fold excess of the L412M-DNA polymerase over singly primed, single-stranded plasmid DNA molecules. Reactions were incubated at 42°C. Reaction products were separated on 0.5 % agarose gels in ethidium bromide. The primer was labeled with ^{32}P so that reaction products could be visualized by exposing the gels to X-ray film.

Fig. 2B illustrates the results of synthesis of plasmid DNA with Rhodamine-dUTP by wild type and L412M-DNA polymerases. Wild-type and the L412M-DNA polymerases were incubated for 5 min (lanes a-d), 30 min (lanes e-h), and 60 min (lanes i-l) at 42°C. Lanes a and b, e and f, and i and j contain reaction products with the L412M-DNA polymerase. Lanes c and d, g and h, and k and l contain reaction products with the wild-type T4 DNA polymerase. At 5 min and at 30 min, reaction products for the L412M-DNA polymerase (lanes a, b, e, f) had lower mobilities and are thus longer than products synthesized by the wild type T4 DNA polymerase (lanes c, d, g, h).

T4 DNA ligase and ATP were added to some of the reactions to measure production of full-length plasmid DNA. The presence of ligase is indicated by a "+" above the lanes; no ligase is indicated by a "-". When primed circular plasmid DNAs are fully replicated, the 3'-end of the synthesized DNA can be ligated to the 5'-end of the primer. Thus, full-length DNA can be seen as DNA which can be converted to covalently closed circular DNA (ccc DNA) by the action of ligase. Covalently closed circular plasmid DNA has a faster mobility than plasmid DNA with gaps or nicks. The mobilities of fully replicated plasmid DNA that has not been ligated and fully replicated ligated DNA are indicated in Fig. 2B. Significantly higher amounts of full-length plasmid DNA (lane i) and covalently closed circular DNA (lane j) are produced by the L412M-DNA polymerase compared to wild-type T4 DNA polymerase (lanes k and l). Longer incubations or increased concentrations of enzyme did not improve the ability of the wild-type T4 DNA polymerase to synthesize full-length plasmid DNA (results not shown). Thus, the L412M-DNA polymerase has superior ability to replicate DNA using rhodamine-dUTP in place of TTP.

Additional bands are apparent, especially in lanes i - l of Fig. 2B. These bands represent sites on the DNA template which are difficult to replicate such as the M13 origin of replication. The L412M-DNA polymerase can more readily replicate past these difficult sites. Similar results have been obtained for the Q380K, E395K, and E743K T4 DNA polymerases.

Reactions with rhodamine-dUTP and rhodamine-dCTP were done for the L412M-DNA polymerase and compared to Sequenase (Fig. 3A). Reactions in lanes a and c contain the T4 L412M-DNA polymerase. Reactions with Sequenase are in lanes b and d. Reactions in lanes a and b were incubated for 30 min at 37°C, reactions in lanes c and d were incubated for 18.5 hours. The L412M-DNA polymerase produced the longest products (lanes b and d).

Example 2

This example demonstrates the importance of glycerol in the reaction mixture (Fig. 3B). The L412M-DNA polymerase (1 pmol) was incubated in reaction mixtures with 67 mM Tris-HCl (pH 8.8), 16.7 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, 6.7 mM $MgCl_2$, 167 μg/ml bovine serum albumin (BSA), 200 μM dCTP, dGTP, and dATP and rhodamine dUTP, and 0.1 pmol primed, single-stranded plasmid DNA. All reactions were incubated at 42°C for 90 min. The reaction products were separated on a 0.5% agarose gel. Lane a is the control and shows the mobility of the primed single-stranded DNA. The reaction mixtures in lanes b and c contained 6.5% glycerol in addition to the above listed reaction components. Lanes d and e contained 7.5% glycerol. Lanes f and g contained 11.25% glycerol. Lanes h and i contained 13.75% glycerol. Lanes j and k contained 16.25% glycerol. Lanes c, e, g, i and k also contained DNA ligase and ATP. As the glycerol concentration was increased, longer fluorophore-labeled DNAs were produced as demonstrated by the increases in full-length (lanes f-j) and cccDNA (lanes i and k).

Example 3

In order to use the L412M-DNA polymerase for the synthesis of complementary fluorophore-labeled DNAs, alone or in combination with accessory proteins and/or the gene 32 protein, the enzyme is directed to one of the two potential sites for DNA polymerase action that exist on each linear duplex DNA. The following procedure is designed so that users will be able to convert long pieces of duplex DNA, tens of thousands of nucleotides in length, to duplex DNA in which one of the complementary strands contains fluorophore-labeled DNA and in which each duplex DNA contains the means to anchor the duplex DNA to a streptavidin-coated bead. The bead-fluorophore DNA complex is immobilized in a flow cell for the digestion and the detection of fluorophore dNMPs which are the next steps of the single molecule sequencing method. The means already exist to immobilize single DNA molecules in a flow cell [Ambrose, *et al.*, *Ber. Busenges Phys. Chem.*, 97:1535 (1993)]. The following

procedure provides a method to synthesize long chains of complementary DNA in a form which can be immobilized in a flow cell in preparation for the digestion and detection steps of the single molecule sequencing method and other DNA sequencing methods which rely on fluorophore-labeled DNA. This method could also be adapted for use in preparing other types of modified DNA, for DNA amplification and for cloning procedures.

First, genomic and/or chromosomal DNA is prepared to minimize breaking of the DNA. Known methods for preparation of high molecular weight DNA, such as immobilization in agarose, may be employed [Ausebel, *et al.*, *Current Procedures in Molecular Biology.*, 1:2.5.11 (1995)]. The next step is to digest the high molecular weight DNA, which may be intact chromosomes, with a restriction endonuclease which cuts DNA only infrequently so that the cut DNA fragments are still for the most part several thousand or tens of thousands of nucleotides in length. The next step is to convert these long duplex DNAs into substrates so that one of the duplex strands can be converted into the complementary fluorophore-labeled strand. A procedure to achieve synthesis of a complementary fluorophore-labeled DNA is depicted in Fig. 4.

The linear duplex DNAs that result from restriction endonuclease cleavages have two complementary ends. In the drawing in Fig. 4A, two 5' overhanging four nucleotide complementary ends are indicated for the model linear duplex DNA. Linear duplex DNAs also have two 3' ends that can be used by DNA polymerases. In order to limit DNA polymerase activity to a single 3' end, one end is blocked by annealing a self-complementary hairpin DNA which has an unpaired end that is complementary to the restriction endonuclease cut linear duplex DNA.

The hairpin-anchor DNA is covalently joined to the linear duplex DNA by DNA ligation (Fig. 4B). One important feature of the hairpin DNA is that this DNA contains one or more biotin residues which are used to anchor the DNA to a streptavidin-coated bead which is required in a later step in the single molecule sequencing method (biotin is indicated by a "*" in Fig. 4).

A further important feature of the hairpin-anchor DNA is that there is a phosphorothioate group in the linkage joining the 3'-terminal nucleotide (the phosphorothioate containing linkage is indicated by an "s" in Fig. 4). When the phosphorothioate linkage is formed, two diasteriomers are made in about equal amounts. One of the phosphorothioate interlinkages is hydrolyzable by the 3'→5' exonuclease activity of T4 DNA polymerase, while the second is resistant [Romaniuk, P., *et al.*, *J. Biol. Chem.*, 257:7684-7688 (1982); Gupta, *et al.*, *J. Biol. Chem.*, 257:7689-7692 (1982)]. The nonhydrolyzable internucleotide linkage protects DNA 5' to the linkage from digestion.

After the hairpin-anchor DNA is joined by DNA ligation to the linear duplex DNA, the resulting joint DNA molecule has a single 3'-end and a single 5'-end (Fig. 4B). The 5'-end has an unpaired DNA sequence complementary to restriction endonuclease cut DNA. Restriction endonucleases are known which cut DNA infrequently so that chromosomal DNA is fragmented into linear duplex DNAs several thousand or tens of thousands nucleotides in length. Hairpin-anchors can be prepared with complementary ends to match these selected nucleases. The hairpin-anchor DNA also has self-complementary sequences with an intervening loop sequence. The self-complementary sequences can vary, but the base pairing between the sequences must be of sufficient stability so that the hairpin structure forms readily under experimental conditions. The loop sequence can also vary, but the loop sequence must not destabilize the hairpin structure and it must contain one or more biotin residues.

The 3'-end of the hairpin-anchor DNA has the phosphorothioate linkage. Existing automated DNA synthesis procedures using phosphoramidite chemistry can be used for the synthesis of the hairpin-anchor DNA. The two phosphorothioate isomers of the hairpin-anchor DNA are produced in about equal amounts from the synthesis. The isomer which is nonhydrolyzable by T4 DNA polymerase can be prepared by treating the mixture of hairpin-anchor DNAs with T4 DNA polymerase under DNA digestion conditions. Only the

nonhydrolyzable hairpin-anchor DNA remains after the digestion reaction is completed.

A variation of the steps depicted in Figs. 4A and B is to join the hairpin-anchor DNA to the linear duplex DNA by blunt-end ligation. Linear duplex DNA can be prepared by restriction endonuclease digestion as above or by other methods that fragment the DNA into large pieces, such as shearing the DNA. The fragmented DNA is then made blunt-ended using standard procedures [Ausebel, *et al.*, *Current Procedures in Molecular Biology*, (1995)] . For this application, a blunt-ended hairpin-anchor is prepared, but this DNA still retains the biotin and phosphorothioate modifications as indicated in Fig. 4A. Blunt-end ligation conditions are then used to join the hairpin-anchor DNA to the linear duplex DNA so that usually only a single hairpin-anchor DNA is joined to each linear duplex DNA. One advantage to this method is that a single universal hairpin-anchor DNA would be sufficient. Another advantage is that it may be useful for some DNA sequencing projects to have methods other than restriction endonuclease cleavage for fragmenting DNA.

The joint hairpin-anchor:linear duplex DNA is then treated with the T4-DNA polymerase under conditions so that the 3'→5' exonuclease activity is functioning, but not the polymerase activity. Selective activation of the 3'→5' exonuclease is achieved simply by not including dNTPs in the reaction mixture. One suitable reaction buffer contains 18% glycerol, 50 to 70 mM Tris-HCl (pH 7.0 to 8.8), 5 to 7 mM $MgCl_2$, 16.7 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, and 0.2 mg/ml bovine serum albumin. Reaction mixtures are incubated between 37°C to 42°C.

After exonuclease digestion the joint hairpin-anchor:duplex DNA is degraded partially or until the enzyme reaches a nonhydrolyzable phosphorothioate linkage. The advantage of preparing a hairpin-anchor DNA with a phosphorothioate linkage is now apparent. If the nonhydrolyzable linkage were not present, DNAs may be degraded so far that the primer is lost. A

primer is required by DNA polymerases for the synthesis of a complementary DNA.

In order to convert the T4-DNA polymerase from a 3'→5' exonuclease to a polymerase, dNTPs are added. If fluorophore-labeled dNTPs are added, the DNA product synthesized by the polymerase is fluorophore labeled. The synthesis of the complementary fluorophore-labeled DNA product by the L412M-DNA polymerase may be enhanced by the addition of T4 DNA polymerase accessory proteins such as the products of T4 genes 44, 45 and 62 and/or the T4 single-stranded DNA binding protein, the product of gene 32. Alternatively, a mixture of DNA polymerases, such as the T4 L412M-DNA polymerase plus Sequenase, may be employed.

Another variation is to use a hairpin-anchor DNA that lacks the biotin residue. Biotin-labeled dUTP is incorporated readily by the T4 DNA polymerase [Langer, P. R., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 78:6633-6637 (1981)]. If biotin-labeled dUTP is incubated for a short time with dATP, dCTP, and dGTP and the L412M-DNA polymerase, the joint hairpin-anchor:duplex DNA is labeled with one or more biotin residues. Joint hairpin-anchor:duplex DNAs with two hairpin-anchor DNAs will not be labeled with biotin; DNAs with no hairpin-anchor DNA will have been digested (Fig. 4C). Streptavidin-coated beads can then be added to extract the biotin-labeled hairpin-anchor:duplex DNAs and to trap unincorporated biotin-labeled dUTP. The unincorporated dNTPs can be washed away. The fluorophore dNTPs can then be added along with the L412M-DNA polymerase and accessory proteins as needed to complete the synthesis of the complementary fluorophore labeled DNA. One potential advantage of using this procedure is that joint hairpin-anchor:duplex molecules with a single hairpin-anchor are selected from the pool of molecules.

An alternative to the steps depicted in Figs. 4A - 4D is to treat linear duplex DNA, with or without a prior fragmentation treatment, with the L412M-DNA polymerase to digest the DNA from both 3'-ends (Fig. 4E). Fluorophore dNTPs are then added along with accessory proteins as needed. The fluorophore

modified duplex DNA is resistant to digestion by most restriction endonucleases, but the unmodified duplex DNA will remain sensitive. Addition of a restriction endonuclease that cuts frequently will likely result in fragmenting the DNA in the unmodified region. The restriction cut ends can then be annealed to a hairpin-anchor DNA with a complementary end. DNA ligation links the hairpin-anchor DNA to the fluorophore-labeled DNA. The resulting DNA molecule resembles the final product depicted in Fig. 4D.

Example 4

Synthesis of DNA probes is similar to synthesis of long fluorophore-labeled DNAs for single-molecule sequencing. The primary difference is that DNA probes are shorter. A second difference is that while a high level of substitution of fluorophore nucleotides for non-modified nucleotides is required for single molecule DNA sequencing, less substitution is required to produce DNA probes with the greatest sensitivity. For example, DNA probes containing a high level of fluorescein-dUMP may be less bright than DNA probes with fewer fluorescein-dUMP molecules because of quenching.

A DNA probe made with rhodamine-dCTP is shown in Fig. 5 (lanes g-i). The reaction conditions were as described for Fig. 2B except that a second oligonucleotide was annealed 300 nucleotides downstream from the primer. The downstream oligonucleotide acts as a block to synthesis and, thus, limits the fluorophore-labeled product to a length of approximately 300 nucleotides.

The reaction conditions are identical to the conditions for Fig. 2B except that reactions contained 200 μM dATP, dGTP and dTTP with 200 μM biotin dCTP (lanes a-c), or 200 μM DIG-dCTP (lanes d-f), or 200 μM rhodamine-dCTP. The reactions were incubated for 5 min (lanes a, d, g), 15 min (lanes b, e, h), and 30 min (lanes c, f, i). The reactions in Fig. 5 contained the L412M-DNA polymerase, but similar results were obtained with the D112A+E114A+L412M-DNA polymerase. A high yield of the 300-nucleotide biotin-

(lanes a-c) and rhodamine- (lanes g-i) labeled probes were obtained. Full-length DIG-labeled probe (lanes d-f) was not obtained under these conditions, but longer reaction times increase the yield of full-length probe.

The amount of labeled nucleotide in the product can be varied by using various ratios of modified and non-modified dNTPs in the reactions. One hundred percent (100%) rhodamine-dCTP was used for the reactions in Fig. 5. Fluorescence intensity can be determined by using a fluorimeter. The fluorescence intensity obtained with 100% rhodamine-dCMP substitution can then be compared with DNAs made with less rhodamine-dCMP to determine the optional degree of substitution.

While there have been shown and described the fundamental novel features of the invention, it will be understood that various omissions, substitutions and changes in the form and details illustrated may be made by those skilled in the art without departing from the spirit of the invention. It is the intention, therefore, to be limited only as indicated by the scope of the following claims.

We claim:

1. In a single molecule sequencing method, wherein a DNA polymerase is used to synthesize a complementary DNA with fluorophore-labeled deoxynucleoside triphosphates, each of the fluorophore dNTPs having a fluorophore tag that can be used to identify the nucleotide, a single fluorophore-labeled DNA is immobilized in a flow cell and subjected to exonuclease digestion, a flow system carrying each released fluorophore-labeled deoxynucleoside monophosphate (fluorophore dNMP) to a highly sensitive fluorescence detector capable of single molecule detection, whereby the order of the fluorophore dNMPs detected gives the sequence of the DNA, the improvement comprising: using as polymerase a mutant bacteriophage T4 DNA polymerase which has increased ability to synthesize accurately long chains of complementary, fluorophore-labeled DNA.

2. A method according to claim 1, wherein the polymerase is selected from L412M-DNA polymerase, Q380K-DNA polymerase, E395K-DNA polymerase, E743K-DNA polymerase, M725I-DNA polymerase, M725V-DNA polymerase, S756P-DNA polymerase, L771F-DNA polymerase, L771H-DNA polymerase, [L771+V]-DNA polymerase, [L771+D]-DNA polymerase, V355A-DNA polymerase, E395K+L412M-DNA polymerase, L412M-E743K-DNA polymerase, E395K-L412M+E743K-DNA polymerase, Q380K+L412M+E743K-DNA polymerase, and mixtures of one or more of the foregoing polymerases.

3. The method of claim 1, wherein the polymerase is a multiple mutant with one or more amino acid substitutions selected from the group consisting of: Q380K, E395K, E743K, M725I, M725V, S756P, L771F, L771H, [L771+V], [L771+D] and V355A.

4. A method according to claim 2, wherein the polymerase is L412M-DNA polymerase.

5. In a method for synthesizing a DNA probe, wherein all or a portion of at least one of the nucleotides used in the synthesis is replaced by a corresponding fluorophore-labeled nucleotide, the improvement comprising: using as polymerase a mutant bacteriophage T4 DNA polymerase which has increased ability to synthesize accurately long chains of complementary, fluorophore-labeled DNA.

6. The method of claim 5, wherein the polymerase is selected from L412M-DNA polymerase, Q380K-DNA polymerase, E395K-DNA polymerase, E743K-DNA polymerase, M725I-DNA polymerase, M725V-DNA polymerase, S756P-DNA polymerase, L771F-DNA polymerase, L771H-DNA polymerase, [L771+V]-DNA polymerase, [L771+D]-DNA polymerase, V355A-DNA polymerase, E395K+L412M-DNA polymerase, L412M+E473K-DNA polymerase, E395K+L412M+E743K-DNA polymerase, Q380K+L412M+E743K-DNA polymerase, and mixtures of one or more of the foregoing polymerases.

7. The method of claim 5, wherein the polymerase is a multiple mutant with one or more amino acid substitutions selected from the group consisting of: Q380K, E395K, E743K, M725I, M725V, S756P, L771F, L771H, [L771+V], [L771+D] and V355A.

8. A method according to claim 6, wherein the polymerase is L412M-DNA polymerase.

9. A method for synthesizing a complementary DNA, from a DNA template, the improvement comprising: using a DNA polymerase with increased intrinsic processivity to synthesize the complementary DNA using one or more modified nucleotides.

10. The method of claim 9, wherein said modified nucleotides are selected from the group consisting of: fluorophore-, DIG-, biotin-labeled nucleotides, and 2-aminopurine.

11. The method of claim 10, wherein the polymerase is selected from L412M-DNA polymerase, Q380K-DNA polymerase, E395K-DNA polymerase, E743K-DNA polymerase, M725I-DNA polymerase, M725V-DNA polymerase, S756P-DNA polymerase, L771F-DNA polymerase, L771H-DNA polymerase, [L771+V]-DNA polymerase, [L771+D]-DNA polymerase, V355A-DNA polymerase, E395K+L412M-DNA polymerase, L412M+E473K-DNA polymerase, E395K+L412M+E743K-DNA polymerase, Q380K+L412M+E743K-DNA polymerase, and mixtures of one or more of the foregoing polymerases.

12. The method of claim 10, wherein the polymerase is a multiple mutant with one or more amino acid substitutions selected from the group consisting of: Q380K, E395K, E743K, M725I, M725V, S756P, L771F, L771H, [L771+V], [L771+D] and V355A.

13. The method of claim 12, further comprising the step of employing the resulting complementary DNA in single molecule sequencing, making DNA probes, or mass spectrometry sequencing.

14. A complementary DNA synthesized from one or more modified nucleotides, by a DNA polymerase with increased intrinsic processivity.

15. The complementary DNA of claim 14, wherein said modified nucleotides are selected from the group consisting of: fluorophore-, DIG-, biotin-labeled nucleotides, and 2-aminopurine.

16. The complementary DNA of claim 15, wherein the polymerase is selected from L412M-DNA polymerase, Q380K-DNA polymerase, E395K-DNA polymerase, E743K-DNA polymerase, M725I-DNA polymerase, M725V-DNA polymerase, S756P-DNA polymerase, L771F-DNA polymerase, L771H-DNA polymerase, [L771+V]-DNA polymerase, [L771+D]-DNA polymerase, V355A-DNA polymerase, E395K+L412M-DNA polymerase, L412M+E473K-DNA polymerase, E395K+L412M+E743K-DNA polymerase, Q380K+L412M+E743K-DNA polymerase, and mixtures of one or more of the foregoing polymerases.

17. The complementary DNA of claim 15, wherein the polymerase is a multiple mutant with one or more amino acid substitutions selected from the group consisting of: Q380K, E395K, E743K, M725I, M725V, S756P, L771F, L771H, [L771+V], [L771+D] and V355A.

18. The complementary DNA of claim 16, wherein the polymerase is L412M-DNA polymerase.

FIG. 1A

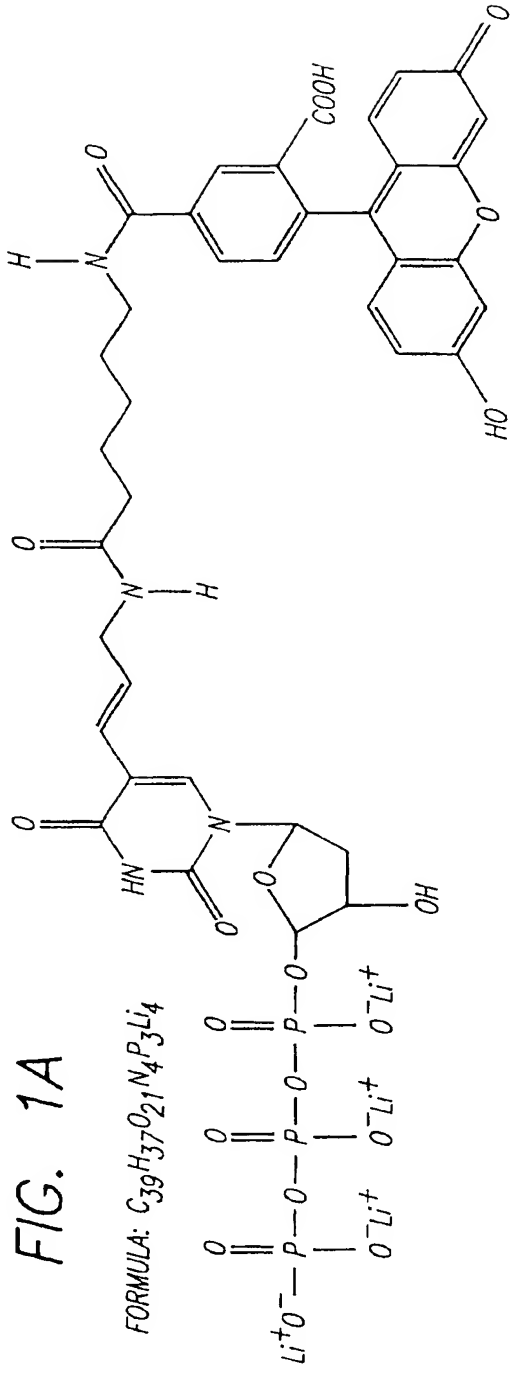
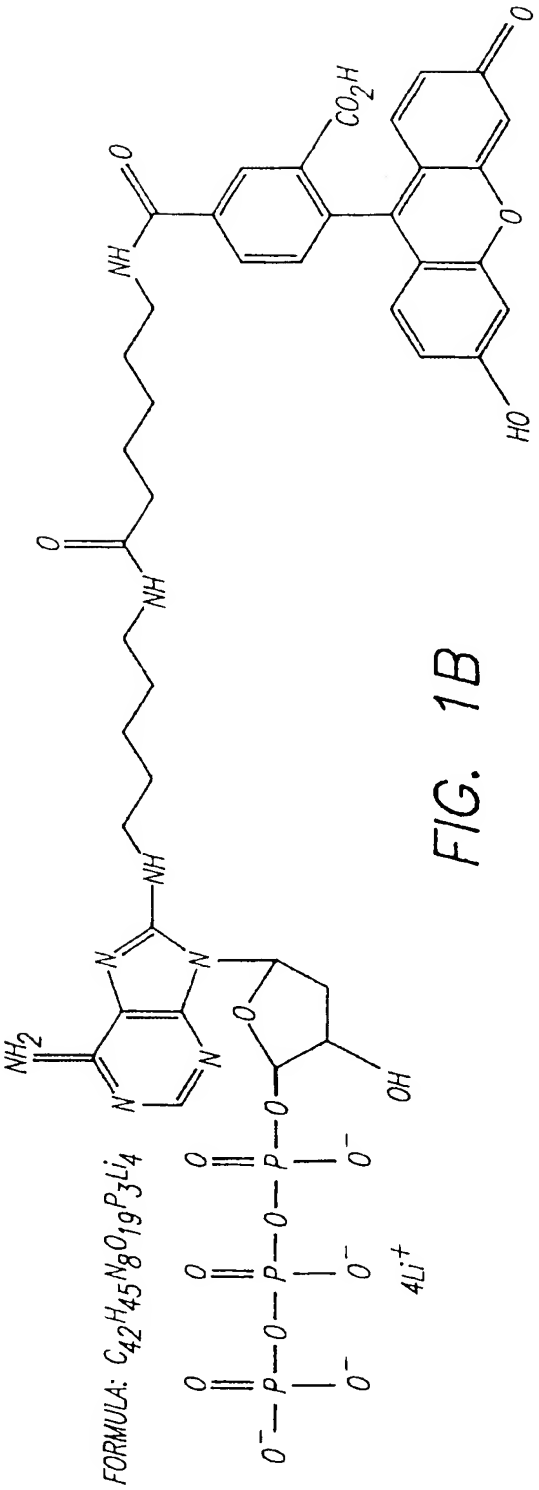


FIG. 1B



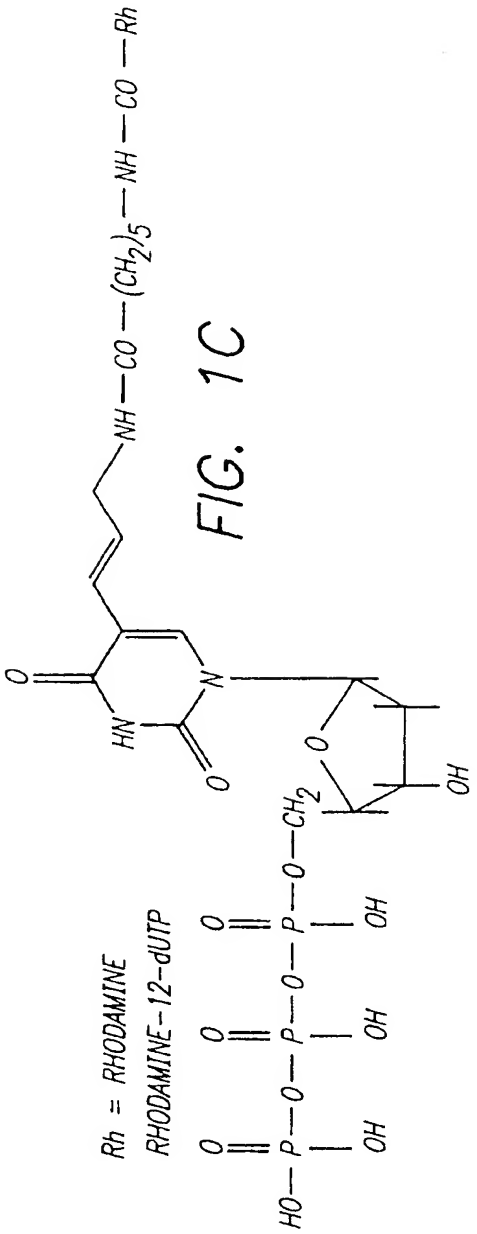

Rh = RHODAMINE
RHODAMINE-12-dUTP
NH — CO — (CH2)5 — NH — CO — Rh
HO — P — O — P — O — P — O — CH2
OH OH OH
HN
O
N
O
OH

FIG. 1C

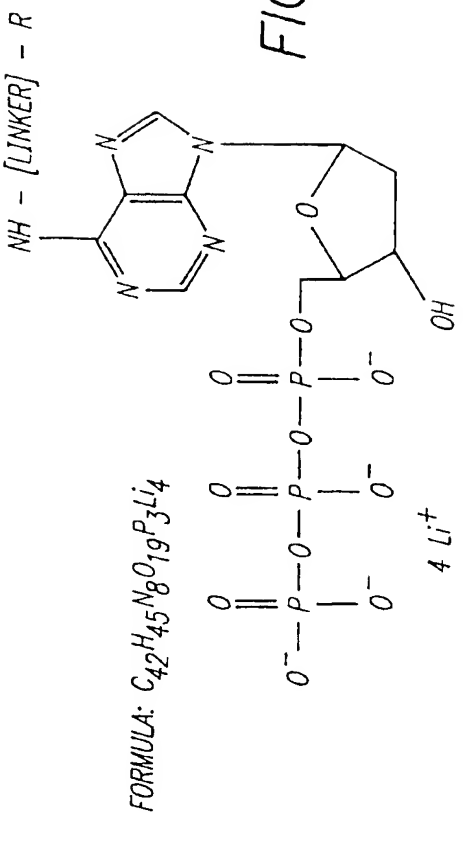
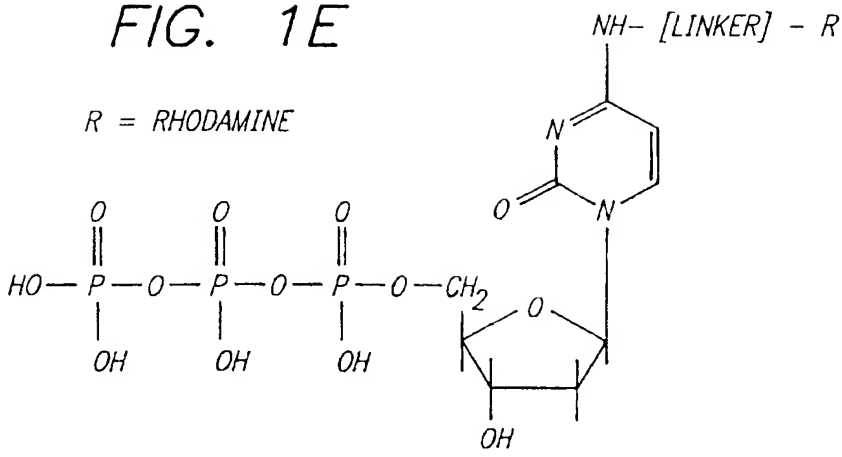

NH - [LINKER] - R
FORMULA: C42H45N8O19P3Li4
O⁻ — P — O — P — O — P — O⁻
O⁻ O⁻ O⁻
4 Li+
OH

FIG. 1D

FIG. 1E


NH- [LINKER] - R
R = RHODAMINE
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O
OH
OH
OH
N
O
N
O
OH

FIG. 1F

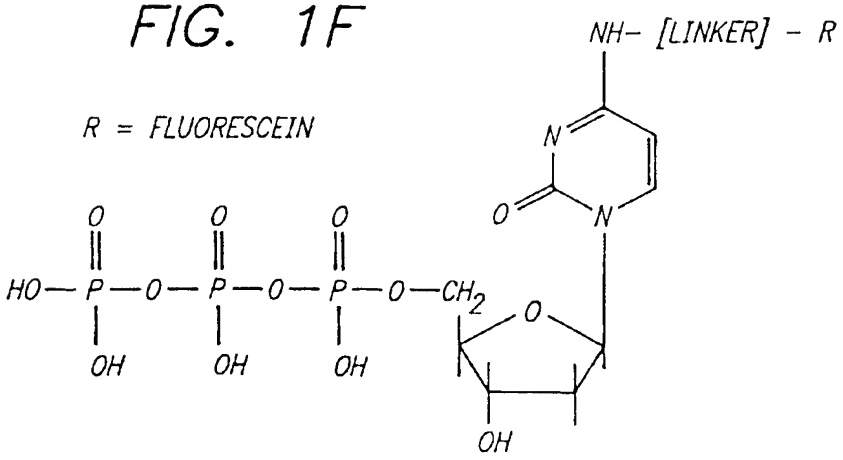

NH- [LINKER] - R
R = FLUORESCEIN
HO— P —O— P —O— P —O—CH2
O
OH
OH
OH
N
O
N
O
OH

FIG. 2A

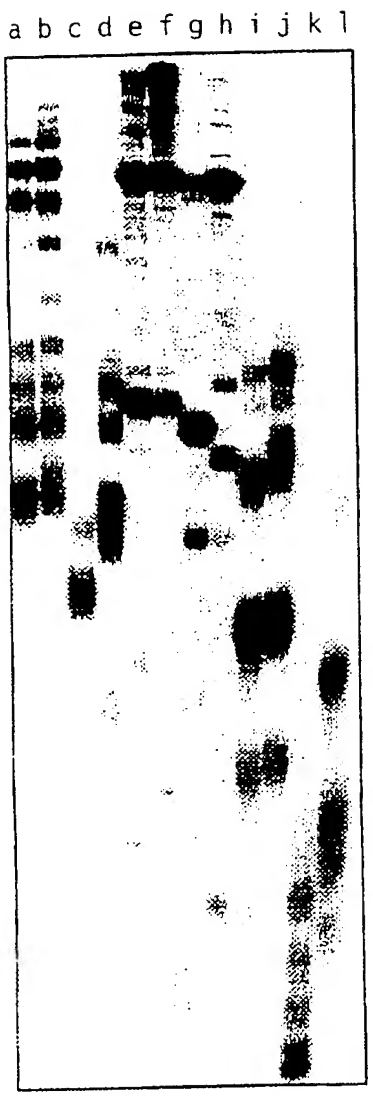


FIG. 3A

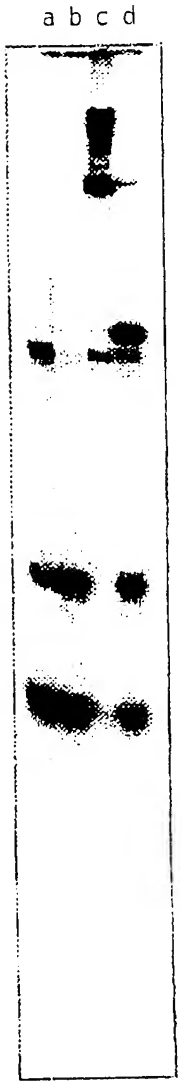


FIG. 2B

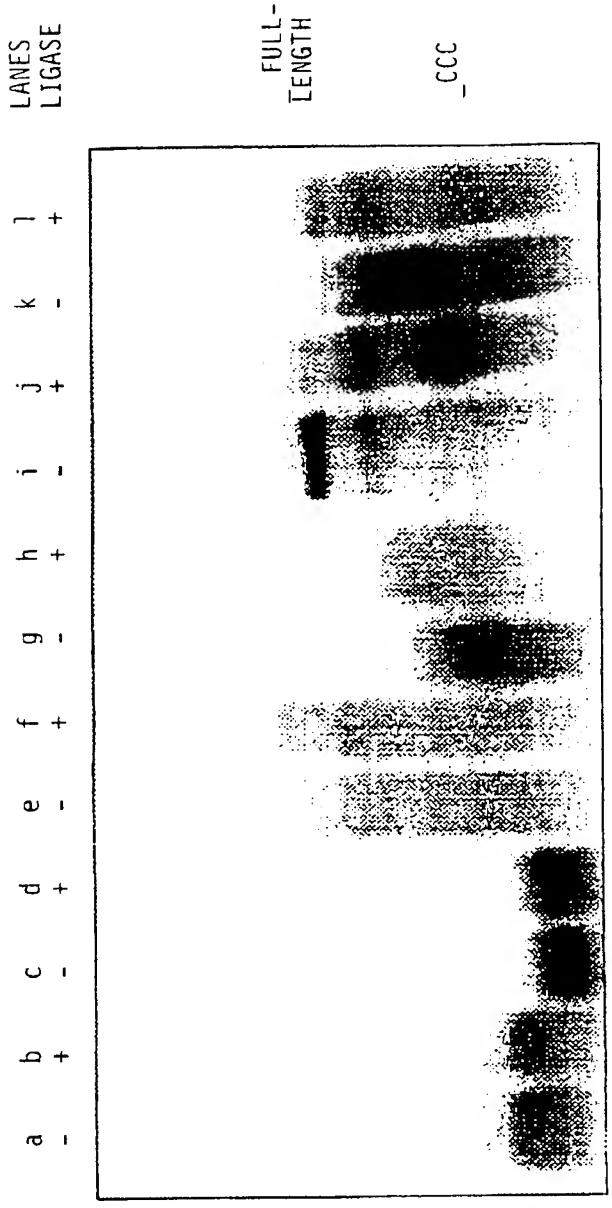


FIG. 3B

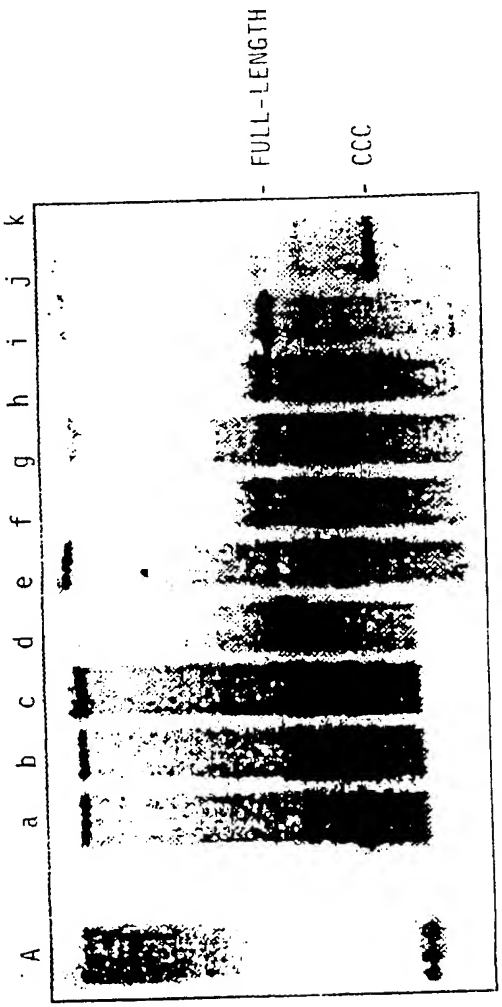


FIG. 4A

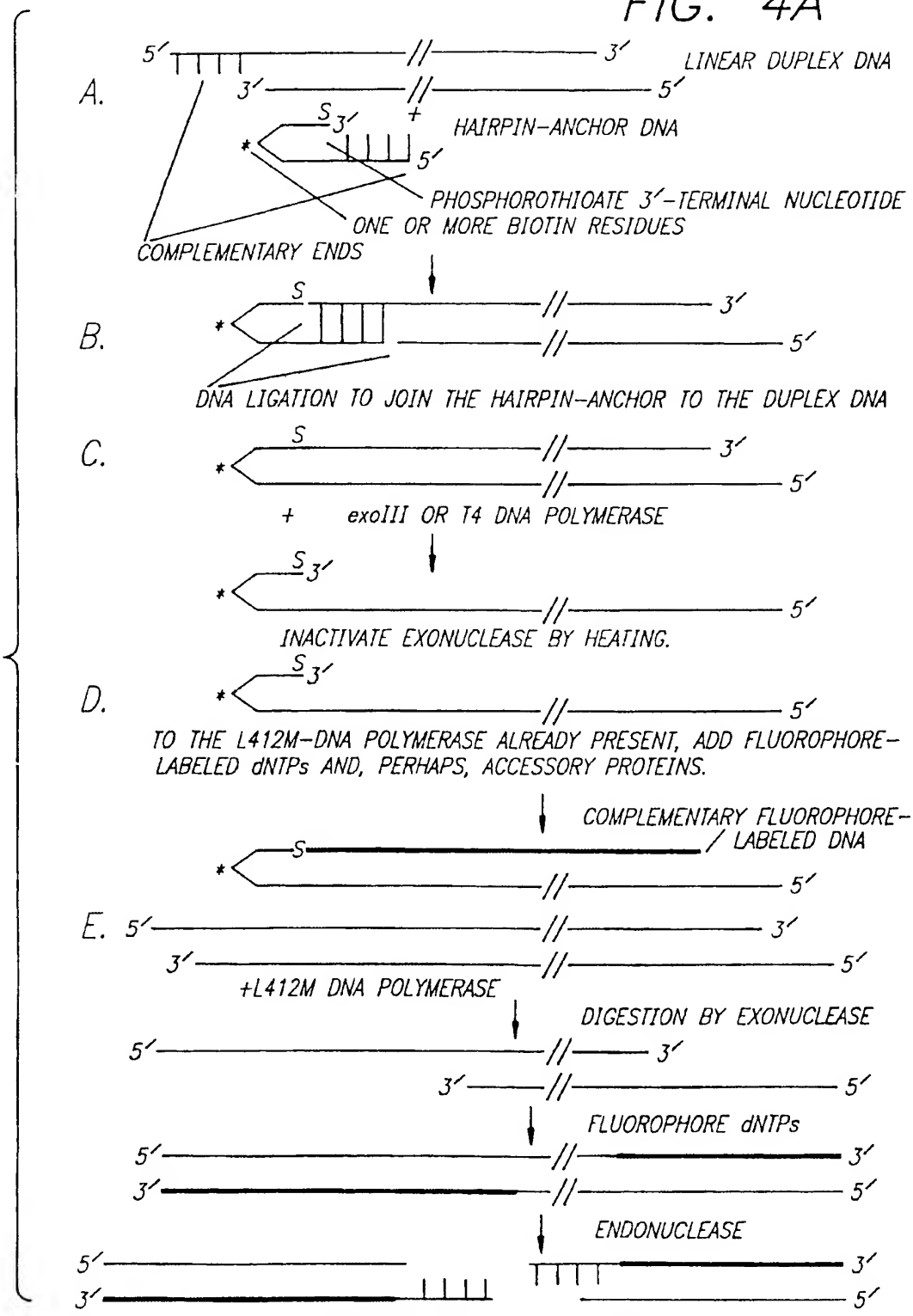
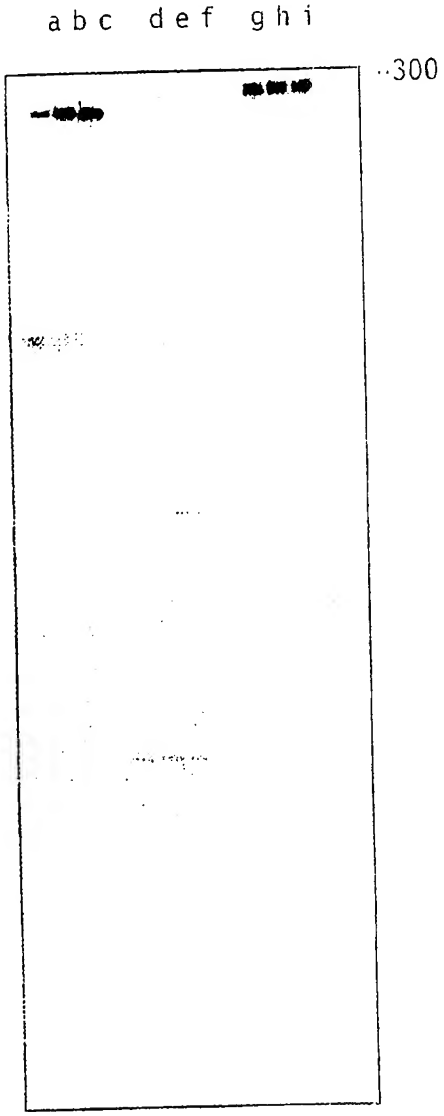

A.
LINEAR DUPLEX DNA
HAIRPIN-ANCHOR DNA
PHOSPHOROTHIOATE 3'-TERMINAL NUCLEOTIDE
ONE OR MORE BIOTIN RESIDUES
COMPLEMENTARY ENDS
B.
DNA LIGATION TO JOIN THE HAIRPIN-ANCHOR TO THE DUPLEX DNA
C.
+ exoIII OR T4 DNA POLYMERASE
INACTIVATE EXONUCLEASE BY HEATING.
D.
TO THE L412M-DNA POLYMERASE ALREADY PRESENT, ADD FLUOROPHORE-LABELED dNTPs AND, PERHAPS, ACCESSORY PROTEINS.
COMPLEMENTARY FLUOROPHORE-LABELED DNA
E.
+L412M DNA POLYMERASE
DIGESTION BY EXONUCLEASE
FLUOROPHORE dNTPs
ENDONUCLEASE

FIG. 5



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/06493

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENET. ANAL. TECHNIQ. APPLICAT., vol. 8, no. 1, 1991, pages 1-7, XP002039495 LLOYD M. D. ET AL.,: "Rapid DNA sequencing based upon single molecule detection"	1,3,5,7, 9,10, 12-15,17
Y	see the whole document	2,4,6,8, 11,16,18

Y	J. VIROL., vol. 67, no. 1, January 1993, pages 60-66, XP002039494 REHA-KRANTZ L. ET AL.,: "Bacteriophage T4 DNA polymerase mutations that confer sensitivity to PPi analog phosphonoacetic acid"	2,4,6,8, 11,16,18
	see the whole document	

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1997	11. 09. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Müller, F

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/06493

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 89 03432 A (US ENERGY) 20 April 1989	1,4,5, 8-10, 13-15,18
Y	see the whole document	2,3,6,7, 11,12, 16,17
Y	WO 95 04162 A (UNIV SOUTHERN CALIFORNIA ;UNIV TORONTO (CA)) 9 February 1995	2,3,6,7, 11,12, 16,17
	see the whole document	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/06493

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